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Preclinical safety evaluation of the ethanolic extract from guavira fruits (*Campomanesia pubescens* (D.C.) O. BERG) in experimental models of acute and short-term toxicity in rats

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ABSTRACT

Campomanesia pubescens is a fruit plant widely distributed in South America and used by the population for medicinal and nutritional purposes, with important economic and cultural value. This study evaluated the toxic potential of the ethanolic extract from *C. pubescens* (EEFCP) fruits through acute and short-term toxicity tests. For the acute toxicity test, female rats received a single oral dose of 2000 mg/kg body weight of EEFCP and were observed for 14 days. In the short-term toxicity test, male and female rats received repeated oral doses of 125, 250, 500 or 1000 mg/kg of EEFCP, being treated and observed for 28 days, and after the treatment period, a satellite and satellite control group remained under observation for another 14 days. No mortality, clinical and organ weight alterations were observed, indicating that LD_{50} is greater than 2000 mg/kg body weight. In addition, the doses tested did not produce significant changes in the behavioral, physiological, hematological or histopathological parameters of animals. These results demonstrate the low acute and short-term toxicity of EEFCP in rats. The data obtained are of great relevance since they provide important information about a plant species of great economic, nutritional and ethnopharmacological value.

1. Introduction

The World Health Organization (WHO) defines as traditional, alternative or complementary medicine the traditional use of plants for maintenance, prevention, diagnosis, improvement or treatment of health, whether in physical or mental diseases, based on beliefs or popular culture (WHO, 2014).

Currently, the interest and use of medicinal plants, phytonutrients or nutraceuticals have been expanding worldwide and many people have used these alternative therapies to treat various diseases in different national contexts (Ekor, 2014; WHO, 2004).

Plants are also widely used as foods around the world as spices, to soften or mask damage to food products, improving flavor and aroma in fresh and/or processed products (Carvalho et al., 2010). The consumers

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CNS, central nervous system; EEFCP, ethanolic extract from *C. pubescens*; HDL, high density lipoprotein; LD_{50}, oral lethal dose; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; OECD, Organization for Economic Cooperation and Development; RDW, red cell distribution width; UDP, Up-and-Down Procedure; UFGD, Federal University of Grande Dourados; WHO, World Health Organization

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of these plants must know the main safety information to avoid health damages due to the uninformed consumption of plants. Previous study has shown that three quarters of herbal preparations marketed do not contain safety information for adequate consumption (Raynor et al., 2011). Despite the widespread use of plants as medicines and foods, often the use of these resources occurs without proper scientific evidence of their pharmacological properties and toxic potential, which is performed through pre-clinical and clinical trials (França et al., 2007; Silveira et al., 2008). Even if a particular species has low toxicity, its inappropriate use associated with risk factors may lead to serious conditions that are sometimes underreported (Brasil, 2012).

Plants of the genus *Campomanesia* belong to the family Myrtaceae and are popularly known as "guavira" or "guabiroba" (Prado and Silva, 2013). There are 37 species distributed in regions from Trinidad to northern Argentina, Andes of Peru, Bolivia, Colombia (Govaerts et al., 2008) and abundantly in the "Cerrado" of the mid-western region of Brazil (Prado and Silva, 2013).

Guavira (*Campomanesia pubescens* (D.C.) O. BERG) is a deciduous shrub that reaches from 1 to 2 m in height. Its flowering occurs between the months of August and September and fruits mature in November/December, presenting juicy pulp with acidulated taste (Lorenzi et al., 2006). Fruits are rounded and green in color when young and yellow after maturation. Guavira presents low energy content due to the reduced concentration of macronutrients, mainly lipids, containing significant levels of calcium, iron, zinc and fibers (Campos et al., 2012). Each 100 g of the fruit has vitamin C concentration of 33 mg, higher than that recommended by the Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO) (Vieira et al., 2006). In addition to being consumed in the fresh form, they are widely used in the preparation of sweets, ice cream, soft drinks, liqueurs and, often, as flavorings in alcoholic distillates (Vallilo et al., 2008), thus characterizing its economic value and sociocultural importance in Latin America. Industries are increasingly exploring the use of guavira fruits for the production of frozen pulps, which increases the interest of both producers and consumers (Kuskoski et al., 2006; Rocha et al., 2011).

Studies have shown that guavira leaves present anti-inflammatory effect due to the reduction of circulating monocytes (Guerrero et al., 2010) and antioxidant effect against the β-carotene/linoleic acid method (Cardoso et al., 2008) and 2-diphenyl-1-picrylhydrazyl (DPPH) (Rocha et al., 2011). In addition, it is also used by the local community for other therapeutic purposes, among them, depurative action, antidiarrheal, reduction of blood cholesterol levels (Ballve et al., 1995), antiseptic of the urinary tract, prevention of rheumatism, liver unblocking (Campos et al., 2012) and as a muscle relaxant through immersion baths (Sousa et al., 2004).

Previous phytochemical studies have demonstrated that the extract obtained from *C. pubescens* leaves have monoterpenes (limonene), sesquiterpenes and triterpenes (Cardoso et al., 2008) and myricitrin (Schmeda-Hirschmann, 1995). In addition, chemical studies with other species of the genus *Campomanesia* demonstrated the presence of the following secondary metabolites: 1) flavanones, chalcones (Pavan et al., 2009), monoterpenes (limonene, α-pinenne and β-pinene) and sesquiterpenes (bicyclogermacrene and globulol) (Coutinho et al., 2009), obtained from *C. adamantium*; 2) Champanones A, B and C (Bonilla et al., 2005), volatile constituents terpenoids, alcohols, carboxylic acids, esters, C13-norisoprenoids, furanic compounds *β*-tricetones, champagnons (Osorio et al., 2006), quercitrin, catechin and tannins (Barbosa, 2009) obtained from *C. lineatifolia*; 3) flavonoids, saponins and tannins obtained from *C. xanthocarpa* (Klafke et al., 2010; Markman et al., 2004).

In view of the expressive consumption of *C. pubescens* fruits, mainly in the form of liqueurs and flavorings in alcoholic beverages as part of the South American culture, and the scarcity of controlled scientific studies that evaluate its toxicity in order to promote safe consumption and consequently the economy from the plant marketing, the aim of the present study was to evaluate the toxic potential of the ethanolic extract obtained from the *C. pubescens* fruit pulp in rats submitted to experimental models of single exposure (acute toxicity) or repeated exposure by 28 days (short-term toxicity).

2. Material and methods

2.1. Botanical material and extract preparation

C. pubescens fruits were collected in December 2015 in Dourados, Mato Grosso do Sul, Brazil (20° 26 ′34 "S and 54° 38′ 47" W), according to authorization issued by the Brazilian Environment Agency (registration number 61621-2 - MMA/ICMBio/SISBIO) and an exsicata (registry Nº. 839) was deposited at the Herbarium of the Federal University of Grande Dourados (UFGD), Mato Grosso do Sul, Dourados, MS, Brazil. Previously crushed fresh fruits (1500.10 g) were macerated with 2 L of ethanol for 7 days. After filtration, the resulting slurry was again extracted with 2 L of ethanol, the union of the liquid fraction obtained after rotavaporation resulted in 334.67 g of ethanolic extract from *C. pubescens* fruits (EEFCP). The product obtained has a semisolid appearance and was diluted in distilled water for oral administration (gavage). Based on the yield of the extract, the dose tested in the acute toxicity study (2000 mg/kg/bw) is roughly equivalent to 9 g of fruit and the highest dose to be tested in the short-term toxicity study (1000 mg/kg/bw) is roughly equivalent to 4.5 g of fruit.

The interest in the ethanolic extract, in particular, arose due to the widespread use of *C. pubescens* fruits as liqueurs and flavorings in alcoholic beverages (where the fruit remains immersed in distilled beverages and alcohol acts as a solvent in extracting compounds that produce the characteristic flavor of these beverages), as an integral part of the culture of countries where the plant is found (Vallilo et al., 2008).

2.2. Quantification of the flavonoid content

For quantification of the flavonoid content, the protocol described by Lin and Tang (2007) was used. EEFCP was solubilized in ethanol at concentration of 1000 μg mL^{-1}. In the test, 1.5 mL of 95% ethyl alcohol, 2.8 mL of distilled water, 500 μL of sample, 100 μL of sodium acetate ($NaC_2H_3O_2{\cdot}3H_2O$) 1 $mol.L^{-1}$ and 100 μL of 10% aluminum chloride ($AlCl_3{\cdot}6H_2O$) were used. After 40 min of reaction at room temperature, readings were performed in spectrophotometer at 415 nm.

For the blank, the same procedure was performed, with sample being replaced by ethanol. To calculate the flavonoid concentration, an analytical curve (2.5, 5.0, 10.0, 20.0, 25.0, 50.0, 100.0 and 125.0 μg) was prepared using quercetin as standard. The experimental procedure performed with the standard was the same used for samples. Data were submitted to linear regression, obtaining the equation of the line, which had its data used in the calculation of real samples. The results were expressed in milligrams of quercetin per gram of extract and tests were performed in triplicate.

2.3. Extract fractionation

The EEFCP extract (50.15 g) was dissolved in water: ethanol (3: 1 v/v) and partitioned with organic solvents (hexane and ethyl acetate). The hexane fraction of EEFCP was fractionated on a chromatographic column using silica gel (0.063–0.200 mm, Merck) in gradient solvent system (hexane and ethyl acetate) and the obtained fractions were analyzed by comparative thin-layer chromatography (TLC, Macherey-Nagel) and 95: 5 v/v hexane: ethyl acetate on elution. Fractions 57–75 were pooled and submitted to purification using preparative thin-layer chromatography (TLC) with 20 × 20 cm plates on silica gel and elution was performed using 85:15 v/v ethyl hexane: acetate, yielding two samples (**F-1**, 1.7 mg and **F-2**, 1.9 mg). The ethyl acetate fraction of EEFCP was fractionated on a chromatographic column using silica gel (0.063–0.200 mm, Merck) in gradient solvent system hexane: ethyl